METABOLIC ASPECTS OF ALUMINIUM TOXICITY AND SOME POSSIBLE MECHANISMS FOR RESISTANCE

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The restriction of some plant species to extremely acid soils is open to a variety of interpretations. It may be argued that the pressure of competition in such environments is less than in those where there are fewer nutritional limitations on plant growth. While this is certainly one explanation of calcifuge behaviour it begs a number of important physiological questions. Since the seedlings of most plants are unable to establish themselves on podsolic soil from heathland (Rorison 1960a; Clymo 1962; Hackett 1964 and Clarkson 1966b), it is clear that calcifuges, which will grow in this soil, must have undergone genetic and physiological adaptation to combat its unfavourable influences. The limitation on plant growth may be due to combinations of deficiencies of major plant nutrients, particularly of phosphorus, and high concentrations of ions which are demonstrably toxic to most plants. Rorison (1960b) showed that aluminium ions exert an inhibitory effect on root growth of non-calcifuge plants in acid soils—a fact well appreciated by agronomic researchers in the U.S.A. much earlier (Ruprecht 1915; Hartwell and Pember 1918), but apparently little considered by ecologists. While the toxic effect of aluminium is not the only adverse influence on plant growth in acid soils, it is an important one which is likely to be of wide occurrence (Hutchinson 1945).

There have been frequent reports in the literature which show that aluminium has inhibitory effects on plant growth, particularly on the growth of roots, but attempts to relate these observations with specific physiological processes are less common. Johnson and Jackson (1964) and Munns (1965) have shown that aluminium has an inhibitory effect on the uptake and translocation of calcium in wheat seedlings and in *Medicago sativa L.* The work of Trenel and Alten (1934), Randall and Vose (1963) and Rorison (1965) indicates that several types of interaction between aluminium and phosphate may occur in the roots of plants. While these effects of aluminium are of interest and importance the results of Rorison (1958, 1960b) and Clymo (1962) indicate that they are unlikely to provide satisfactory explanations of root failure in aluminium-treated plants.

Evidence presented in this paper indicates that cell division is a primary site of disturbance by aluminium and some suggestion is made of the stage in the mitotic cycle where aluminium acts. There are also more general metabolic changes brought about by aluminium which influence sugar phosphorylation and some consideration is given to the relationships between these effects.

RESULTS AND DISCUSSION

Effects on cell division and DNA synthesis

A convenient starting point for this discussion is to consider the abnormal morphology in a root of *Agrostis tenuis* which has been grown in a dilute solution of aluminium. Where plants are grown in a water culture without aluminium the lateral roots usually are of a smaller diameter than the main axes, and do not develop near to the root tip. The aluminium-treated root in Fig. 1 presents a different picture. Here the main axis and the laterals of different orders are of similar diameter and are developed close to the apical meristems, the whole resembling corralloid roots with mycorrhizal associations. This pattern of development may be reconstructed as follows: failure of the main axis I was followed by initiation of first order laterals IIa and IIb which in their turn ceased to develop, giving rise to secondary axes IIIa and IIIb. This process may continue to give rise to third and fourth order laterals in axes of decreasing length. The consequences of this type of development can be lethal for seedlings under natural conditions since the failure of their roots to penetrate the soil to any depth can eventually lead to their death by desiccation. This growth form strongly suggests that aluminium inhibits the development of the root apex and may have an effect on cell division.

To investigate this matter experiments were made with the adventitious roots developing from the bulbs of onion, *Allium cepa*, which provide convenient material for studying rates of root growth in relation to cell division. Prior to their use in experiments, onion roots were grown in an acid culture medium at pH 4·0, in which the following salts were supplied in mM concentrations $CaCl_2$ 2·5; KCl 0·5; NH_4NO_3 1·0; $MgSO_4$ 0·25; KH_2PO_4 0·1; ferric citrate 0·04 plus minor elements as listed in Hewitt (1952). Aluminium sulphate was added to this solution in concentrations of 10^{-3}–10^{-5} M and the pH adjusted to 4·0 with potassium hydroxide. Measurements of root elongation were made in specially designed chambers, which will be described elsewhere, and the abundance of mitotic figures in aceto-carmine squashes from root meristems was estimated using a technique described in Clarkson (1965).

The effect of aluminium at various concentrations on vigorously growing onion roots is shown in Fig. 2. Six to eight hours after the addition of 10^{-3} M aluminium sulphate the rate of root elongation was reduced to almost zero: if the aluminium solution was removed at this point, and replaced by aluminium-free culture, elongation of the root did not recommence over a period of 36–48 hr, indicating that the effect of aluminium at this concentration is irreversible. The rate of elongation in roots treated with 10^{-4} M aluminium sulphate approached zero after 8–12 hr of treatment, while 10^{-5} M aluminium sulphate caused only a slight decrease in root elongation in comparison with the untreated control. Counts of mitotic figures in root apices were made during similar experiments and are shown in Fig. 3. In the 10^{-3} and 10^{-4} aluminium sulphate treatments there is a similar time course for the inhibition of mitosis and the decline in the rate of root elongation. It is particularly important to note that the aluminium

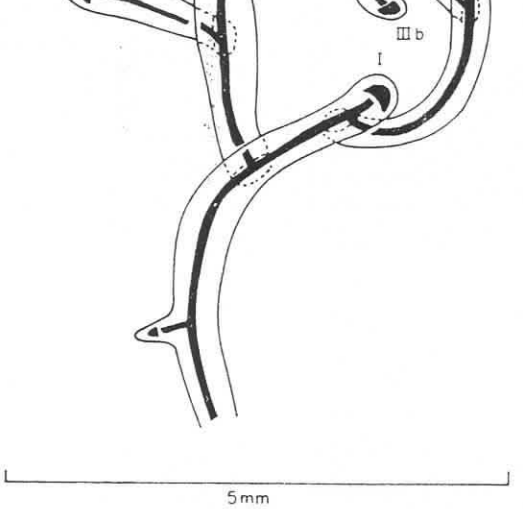

FIG. 1. Diagram of abnormal root branching in a seedling of *Agrostis tenuis* grown for several weeks in a culture solution containing 10^{-3} M aluminium sulphate.

treatments do not seem to arrest mitosis itself since there was no accumulation of mitotic figures; the dividing cells pass into interphase apparently unchecked. Partial inhibition of elongation was achieved if roots were treated with 10^{-3} M aluminium sulphate for periods of time less than 6 hr and then replaced in aluminium-free cultures. If, for instance, roots were treated for 3 hr the rate of elongation decreased by approximately a half of its former value, but thereafter remained constant for at least 36 hr (Fig. 2, broken line). Fig. 4 summarizes a number of experiments of this kind and indicates a linear relationship between the duration of aluminium treatment (in the range 0–6 hr) and subsequent rate of root elongation.

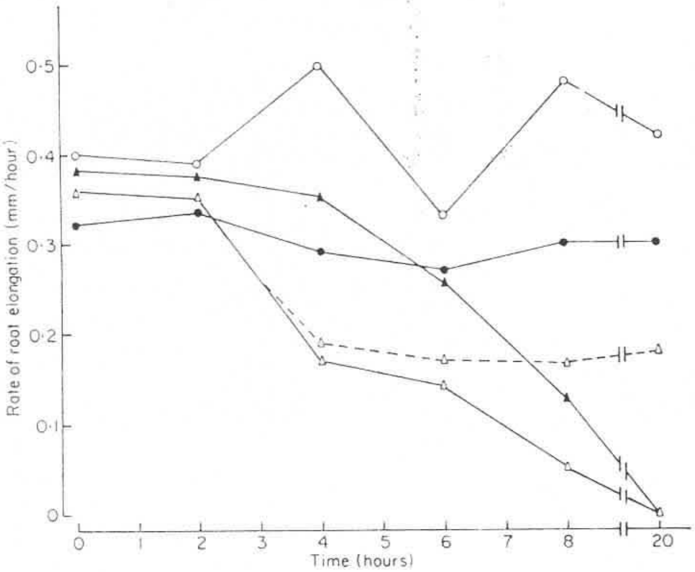

FIG. 2. Elongation of onion roots treated with various concentrations of aluminium sulphate. Open circles, control roots not treated with aluminium; solid circles, 10^{-5} M $Al_2(SO_4)_3$; solid triangles, 10^{-4} M $Al_2(SO_4)_3$; open triangles, 10^{-3} M $Al_2(SO_4)_3$, solid line continuous treatment, broken line, treatment terminated at hour 3.

This time-course of events would be expected if meristematic cells were vulnerable to the influence of aluminium at some specific stage of the mitotic cycle. The partial inhibition of root growth and cell division by short periods of aluminium treatment would depend on the fact that cells do not divide synchronously, thus they would not be at the vulnerable stage at the same time, and also that aluminium is quickly immobilized in the root by binding so that it is unable to move from one site to another.

Some suggestion of the stage in the mitotic cycle where injury occurs can be made by considering the present results jointly with the observations of Mäkinen (1963) and Van't Hoff (1965) on the length of the mitotic cycle in *A. cepa*. The speculative nature of the following discussion is stressed.

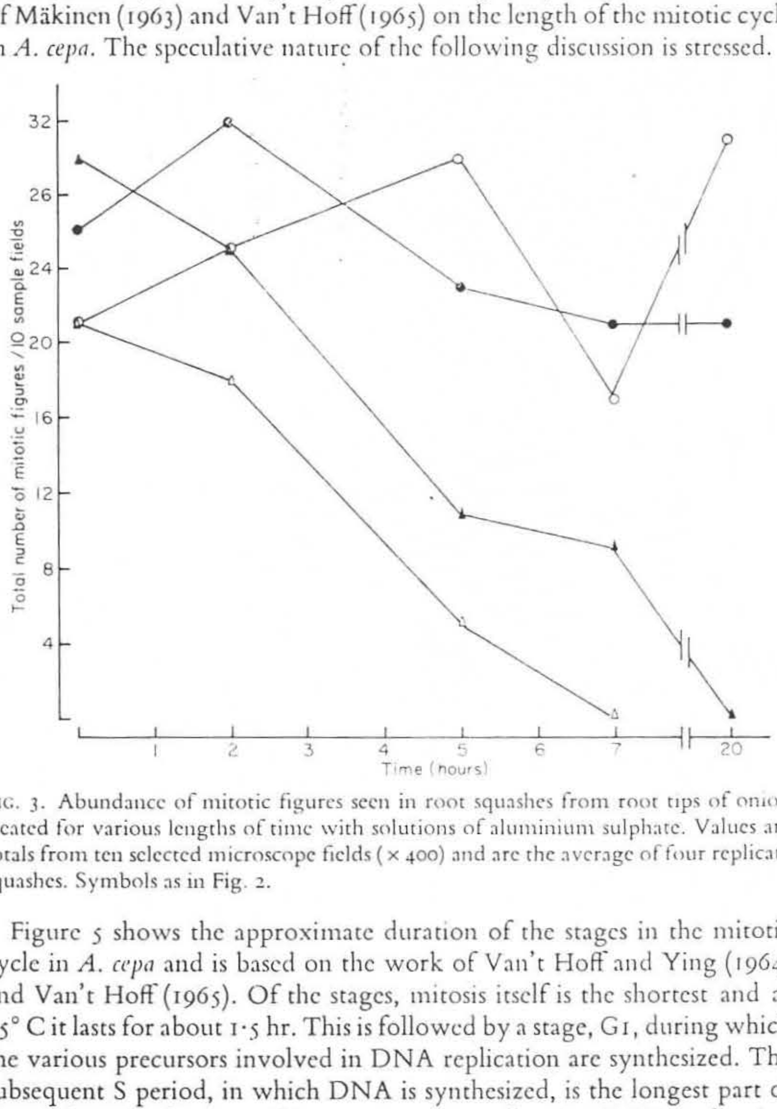

FIG. 3. Abundance of mitotic figures seen in root squashes from root tips of onion treated for various lengths of time with solutions of aluminium sulphate. Values are totals from ten selected microscope fields (× 400) and are the average of four replicate squashes. Symbols as in Fig. 2.

Figure 5 shows the approximate duration of the stages in the mitotic cycle in *A. cepa* and is based on the work of Van't Hoff and Ying (1964) and Van't Hoff (1965). Of the stages, mitosis itself is the shortest and at 25° C it lasts for about 1·5 hr. This is followed by a stage, G1, during which the various precursors involved in DNA replication are synthesized. The subsequent S period, in which DNA is synthesized, is the longest part of the cycle and takes something over 10 hr. Following DNA replication

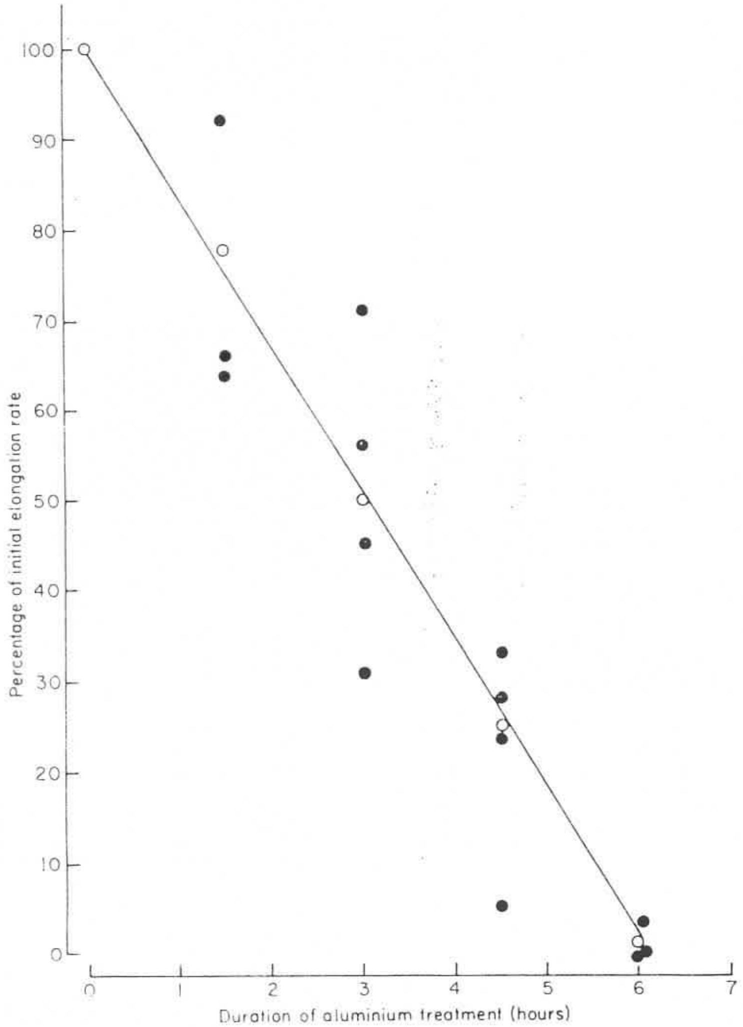

FIG. 4. Rate of root elongation after various periods of treatment with 10^{-3} M $Al_2(SO_4)_3$. Rates expressed as a percentage of that measured before treatment commenced. Open circles are average values, solid dots represent range in replicates.

there is a delay, G2, before the cells enter division. If it is assumed that the time which aluminium takes to reach its site of action, when roots are immersed in 10^{-3} M aluminium is so short that it can be ignored, it would be expected that mitotic figures would continue to be visible for at least

15 hr if aluminium caused a blockage in the G1 period. Blockage at the end of the S-period would, however, eliminate most mitotic figures within 5 hr, whereas a blockage in G2 would reduce this period to no more than 1·5–2·5 hr and would result in the formation of polyploid nuclei. The observations in the present study show that mitosis and root growth are inhibited after 6–8 hr and this timing is consistent with disturbance during the S-period of the mitotic cycle. Since DNA replication occurs in this period, aluminium should inhibit nucleic acid synthesis; evidence in this direction has been provided by Sampson, Clarkson and Davies (1965) who studied the incorporation of radioactive phosphorus into the DNA of barley roots where cell division had been stopped by treatment with aluminium sulphate. Table 1 shows that two distinct fractions of DNA can be distinguished in both aluminium treated and untreated barley roots. One

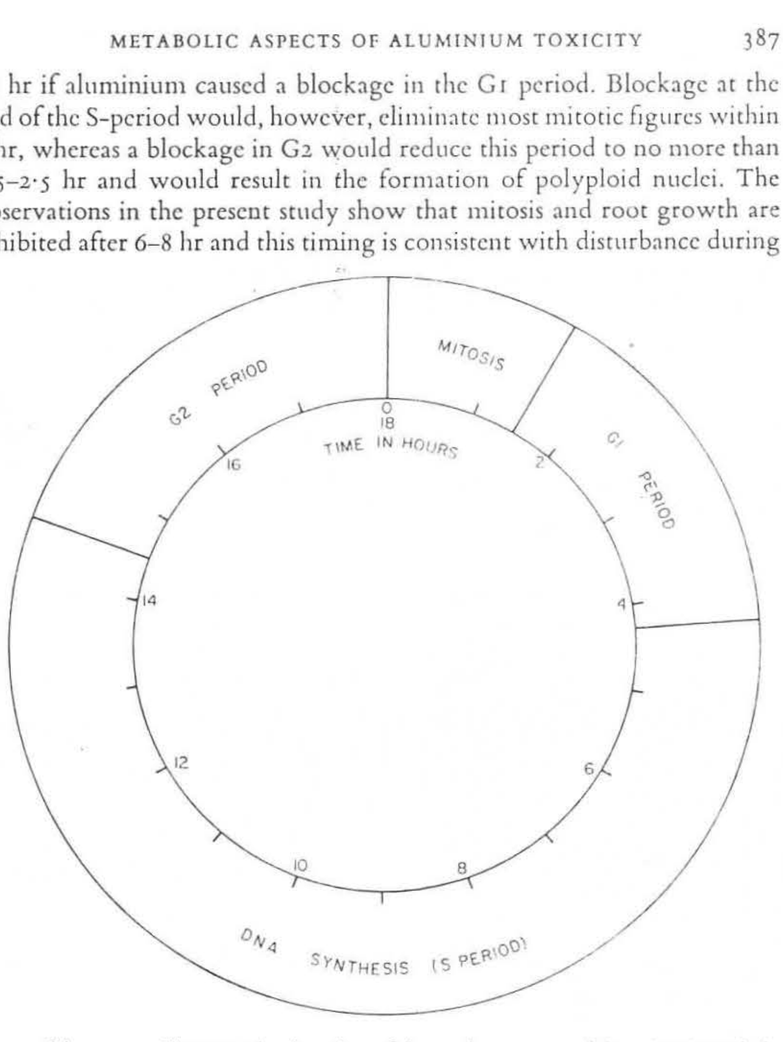

FIG. 5. Diagram to illustrate the duration of the various stages of the mitotic cycle in *Allium cepa*. Derived from Van't Hoff and Ying (1964) and Van't Hoff (1965).

of these fractions is the usual 'genetic' DNA which is stable and has a high molecular weight, the second is a DNA of lower molecular weight which is metabolically labile and is found characteristically in young actively growing tissue (Sampson and Davies 1966). In the untreated roots of the control ^{32}P was incorporated into both of these fractions to produce high specific activities during a 4-hr incubation, but in aluminium-treated roots only one fraction, the low molecular weight fraction, contained significant levels of ^{32}P, the activity in the genetic fraction not being high enough to distinguish it from contamination during the preparative procedures.

TABLE I

Amount and specific activity of DNA-P fractions from aluminium-treated and untreated barley roots after labelling for 4 hours with carrier free ^{32}P. Compiled from Sampson, Clarkson & Davies (1965).

Treatment	DNA fraction	Amount (μg) DNA-P	Radioactivity counts/sec/μg DNA-P
Control −Al	Genetic†	12·8	6·0
+Al		18·5	1·2
Control −Al	Labile‡	3·4	23
+Al		4·8	16

† Genetic DNA with molecular weight, 4–6 × 10^6 and with a mole percentage of Guanine + Cytosine of 42 (Sampson *et al.* (1963)).

‡ Labile DNA with molecular weight 2–3 × 10^5 and with a mole percentage of guanine + cytosine of 52.

These findings are of relevance to the present discussion for they show that (a) there is a failure of genetic DNA synthesis so that the cells cannot pass through the S-period, (b) low molecular weight DNA is synthesized in similar amounts and at similar rates in treated and untreated roots, indicating that nucleic acid metabolism as a whole is not disturbed by aluminium. This latter finding is supported by evidence which shows that aluminium treatment of barley roots had only minor effects on the amount and specific activity of RNA labelled by ^{32}P (Clarkson 1966a). It thus appears that cell division is much more readily disturbed by aluminium than metabolism in general.

Effect of aluminium on phosphorus metabolism and respiration

In contrast with the results so far considered there are a number of more general effects of aluminium on metabolism. Wright (1943) and Wright and Donahue (1953) have advanced the view that the toxic effect of aluminium is an indirect one and is mediated through an internal precipitation of aluminium phosphate in the root which induces phosphorus deficiency. It is difficult to believe that this process could explain the observations on root elongation and cell division, since it was shown that the presence or absence of phosphorus in the aluminium treatment had no effect on the inhibition of mitosis, neither could this inhibition be overcome by treating the root subsequently with phosphorus in the absence of aluminium for periods of up to 1 week (Clarkson 1965). There have been reports, however, which suggest that aluminium may have more subtle effects on phosphorus metabolism (Ragland and Coleman 1962; Randall and Vose 1963; Rorison 1965).

The full significance of interactions between aluminium and phosphorus are not apparent unless the individual phosphorus fractions in the root are examined. This question was studied using barley seedlings pre-treated for 24–48 hr with 10^{-3} M aluminium sulphate and then immersed in solutions of potassium dihydrogen phosphate labelled with ^{32}P (Clarkson 1966a). The effect of aluminium was to increase the amount of phosphorus in the roots, but all of the additional phosphorus was present as inorganic phosphate (Table 3, line 6), and was found to be readily exhangeable with non-radioactive P (Table 2). This suggests that the reaction between aluminium and phosphorus is superficial and occurs either at the root surface or in the free space of the root. Further evidence that this reaction between aluminium and phosphate does not depend on cellular metabolism is shown by the fact that low temperature and toxic levels of dinitrophenol have little effect on the accumulation of extra phosphorus in aluminium treated roots (Table 2). Surface fixation of phosphate is likely to reduce the amount of phosphorus available for translocation to the shoot, and this may be particularly important in situations, like heathland, where phosphorus in the soil solution is already at a very low concentration.

There are, in barley roots, effects on phosphorus metabolism which clearly must occur *within* the cells. If we compare the amount of phosphorus which is not readily exchangeable in the treated and untreated roots (Table 2, column 5) we see that in each case it is similar. Generally this may be taken as the phosphorus that is within the cells. The total phosphorus incorporated into readily soluble organic compounds is shown in Table 3

TABLE 2

Incorporation of phosphorus by barley root segments pretreated in presence or absence of 10^{-3} M $Al_2(SO_4)_3$. Values are nano moles $P(10^{-9})$/g (fresh weight) and are means of 3 replicates ± standard deviation. From Clarkson (1966a)

Conditions	Sample (min)	Treatment	Total P	Non-exchangeable P	Exchanged P
25° C	2	Control −Al	16·0 ± 2	14·0 ± 1·8	2
		+Al	97·0 ± 8·5	18·0 ± 1·8	79
	60	Control −Al	402·0 ± 47·0	399·0 ± 51·9	3
		+Al	588·0 ± 76·3	365·0 ± 48·5	223
25° C	2	Control −Al	8·7 ± 1·2	0	8·7
		+Al	28·2 ± 4·1	0	28·2
$+10^{-3}$ M DNP					
	60	Control −Al	34·3 ± 3·6	2·2 ± 0·1	32·1
		+Al	129·6 ± 14·7	7·1 ± 0·2	122·5
1° C	2	Control −Al	0·6 ± 0·1	0·2	0·4
		+Al	32·7 ± 5·1	0·7 ± 0·2	32·0
	60	Control −Al	12·0 ± 2·4	2·9 ± 0·3	9·1
		+Al	179·3 ± 18·2	2·5 ± 0·3	176·8

TABLE 3

Incorporation of ^{32}P into phosphorylated compounds in whole barley roots after 20 and 100 min. Values are in counts/second/root. From Clarkson (1966a)

Spot	20 min		100 min	
	Control	Aluminium	Control	Aluminium
UTP	7	10	10	14
ATP	19	29	37	56
ADP	4	6	11	9
Hexose-(P)	58	21	161	66
Total organic P	101	79	271	180
Inorganic P	237	804	524	1304
Total	338	883	795	1490

Plants pretreated in an acid water culture, pH 4, with or without 10^{-3} M aluminium sulphate. Root material killed and phosphorus compounds extracted in a mixture of methanol, chloroform and formic acid at −80° C and 1° C respectively. ^{32}P labelled compounds in this extract located on chromatograms by autoradiography, and counted directly.

(line 5) and it is clear that some reduction in this fraction is caused by aluminium. More interesting than this is the partition of phosphorus between a number of important metabolic intermediates. The most striking examples of this are shown in Table 3, where, at sampling periods at 20 and 100 min the amount of ^{32}P incorporated into phosphorylated hexose sugar is much reduced, while there is an apparent accumulation of nucleotide triphosphates, particularly of ATP and UTP.

What are the implications of this disturbed pattern of phosphorus distribution? Hexose phosphates, particularly glucose-6-phosphate, are the entry point of carbohydrate substrates into the respiratory process, and hence a reduction in the rate of their formation, or their amount, will have far reaching consequences on metabolism. Glucose becomes phosphorylated in a reaction with ATP in which hexokinase is the enzyme (Saltman 1953) and the products are glucose-6-phosphate and ADP. Aluminium treatment did not limit this process by reducing the rate of formation of ATP, since, in Table 3 it is clear that this actually increased, as did the total amounts of ATP in the root; in this connection it is interesting to note that Woolhouse (this volume) has shown that acid phosphatases and ATP-ase are inhibited by aluminium. Thus aluminium must act either directly or indirectly to prevent the utilization of ATP in glucose phosphorylation. This inhibition should be reflected by a decrease in respiration, and, indeed, this has been found to be so in several varieties of barley (Table 4), but only after extended aluminium treatment.

If the failure of cell division is not due to the direct action of aluminium on the process, but results, secondarily, from impaired respiratory metabolism, the decrease in oxygen uptake should either precede or at least occur at the same time as the inhibition of cell division. Sampson, Clarkson and Davies (1965) showed that inhibition of cell division in barley root apices is at an advanced stage 6–10 hr after the commencement of aluminium treatment. However, only trifling decreases in the rate of oxygen uptake by barley roots can be detected after 8–11 hr of treatment (Table 4); only after 24 hr was an appreciable reduction found. Similar disparities between the onset of mitotic inhibition and the depression of respiration have been found using onion roots (Clarkson, unpublished data). These results make it seem unlikely that impaired respiratory metabolism is the cause of the inhibition of mitosis. But reduced rates of respiration may account for the secondary effects on the uptake of calcium and of other ions, since these processes are closely linked with respiratory metabolism. In contrast with the present results with onion and barley, Norton (1967 and personal communication) has found decreases in oxygen uptake in the roots of the calcicole sainfoin (*Onobrychis sativa*). A depression of 5% was detected within 30 min of the addition of 2×10^{-3} M aluminium and this increased to around 35% in 3 hr. Inhibition of oxygen uptake and carbon dioxide production was also detected in intact embryos of sainfoin before the onset of cell division. There were no comparable effects on the respiration of the calcifuge Lupin (*Lupinus luteus*). It would seem that further experiments are necessary to establish unequivocally, cause and effect in this work.

TABLE 4

Oxygen uptake by excised roots of barley seedlings pre-treated for various periods with 10^{-3} M $AlCl_3$.

Values are μl O_2 taken up by 1 g root tissue (fresh weight) per hour. Percentage inhibition due to aluminium shown in brackets.

Variety	Pre-treatment	Pre-treatment period (hr)				
		3	5	8	11	24
Himalaya	Control − Al	1099	1113	1086	1097	1009
	+ Al	1021(7)	1012(9)	1007(7)	941(14)	628(38)
Proctor	Control − Al		652			771
	+ Al		632(3)			431(45)
Dayton	Control − Al	635		639		600
	+ Al	710(+11)		602(6)		497(17)
Kearny	Control − Al	762		727		719
	+ Al	772(+1)		698(4)		673(6)

Seedlings pretreated in an acid culture solution, pH 4, with or without 10^{-3} M $AlCl_3$. Excised roots incubated in respirometer flask in the presence of 1% sucrose, pH 4·0, with or without 10^{-3} M $AlCl_3$. Oxygen uptake measured in a Gilson differential respirometer.

Resistance to aluminium toxicity

From the ecologists viewpoint the nature of resistance to aluminium toxicity may be of more interest than the mechanism of the toxicity itself. At this time it is possible only to speculate on resistance but the justification for doing so is that it may suggest lines for future work.

Difficulties in envisaging a method of resistance arise because the system must be specific for aluminium. Preliminary findings (Clarkson, unpublished) suggest that resistance to aluminium in *Agrostis setacea* and rye *Secale cereale*, does not confer on these plants resistance to other trivalent metal ions of similar chemical nature, e.g., gallium, scandium, yttrium and lanthanum, even though they may act on cell division in the same way as aluminium (Clarkson 1965). This problem of metal specificity is also discussed by Turner (this volume). Taking the most direct approach, we may propose that the resistant species prevents aluminium reaching its site of action either by preventing it entering the cell, or by waylaying the aluminium once it is within the cell.

The biochemical evidence in this paper suggests that there may be at least two sites within the cell where aluminium acts, viz. the nucleus and the mitochondria where respiratory disturbances are likely to have their origin. An intracellular resistance mechanism might prevent the aluminium reaching these sites either by providing alternative binding sites of higher affinity which preferentially accumulate aluminium without damaging metabolism, or by rendering the ion into a non-toxic form by chemical modification. Where differentiated cells possess a vacuole, it is easy to visualize this as a depository for unwanted metals, however, meristematic cells are not vacuolated in either resistant or tolerant species and it seems that aluminium is particularly disruptive in these cells. Almost any charged surface within the cell might be expected to bind aluminium, but no suggestion can be offered of one which would do so preferentially. Chemical modification of aluminium by chelation with EDTA is known to reduce its toxicity greatly and to promote its mobility within the plant (Clymo 1962). It would seem, then, if the cell could act in some way to prevent the aluminium being in a cationic form it can avoid injury; as aluminate anion it is accumulated in quantity, and apparently innocuously by calcicole plants, Jones (1961). If such a specific chelating agent exists within the cell it has thus far escaped detection.

There is evidence that a large proportion of the aluminium in barley roots is associated with the cell wall where it may be present as an amorphous precipitate of $(Al(OH)_3)_n$ (Clarkson 1967). This precipitation is brought about by the hydrolysis of aluminium ions by hydroxyl ions at the root surface. A resistant species might produce hydroxyl ions at its root surface rapidly enough to precipitate all the incoming aluminium in this way. Vose and Randall (1962) suggested that low root cation exchange capacities are associated with tolerance to aluminium and manganese in some varieties of *Lolium perenne*, and more recently Foy *et al.* (1967) have expanded this idea. Some interesting studies by Foy, Burns, Brown and Fleming (1965) have shown that an aluminium-resistant variety of wheat, Atlas 66, was able to increase the pH of the soil and water cultures in which it was grown, towards more neutral values, causing removal of aluminium from solution by precipitation. A non-resistant variety, Monon, lacked this ability or showed a tendency to make its cultures more acid.

These differential pH effects appeared to be partially dependent on the balance between nitrate and ammonium ions in the culture solution, and suggest that nitrate removal from the solution is more rapid in Atlas 66 than in Monon (Foy *et al.* 1967). Where ammonium ions are appreciably more abundant than nitrate the differential pH changes were not observable (Foy, personal communication). In relating these results to field situations it is as well to remember that in most podsolized soils, where resistance to aluminium toxicity may determine the ability of a species to establish itself, the nitrate concentration in the soil is very small in relation to the ammonium (Richardson 1938).

Magistad (1925) showed that small changes in pH in the range 4·0–4·5 will produce large, and physiologically significant, changes in the solubility of aluminium in water. The ability of plants to effect such changes in the vicinity of their roots may be the basis of varietal differences in response to soil acidity and other factors, and merits the closest attention.

It is not immediately apparent how general properties of the root such as its cation exchange capacity would permit discrimination between aluminium and, say, scandium, but specificity may be dependent on more subtle features, for instance, the spatial distribution of hydroxyl ions at the root or cell surface.

CONCLUSIONS

When aluminium reaches its site of action within cells of the root it causes disordered metabolism which results in either the total or the partial failure of cell division probably by interference with DNA synthesis in the S period of the mitotic cycle. This results in the inhibition of root growth and the development of abnormal root morphology. Disturbances in phosphorus metabolism may be detected at two levels, the first at the root surface where a reaction with aluminium apparently fixes phosphate, and the second is within the cell where the distribution of phosphorylated intermediates is altered in such a way as to depress respiratory metabolism. There is also evidence that aluminium may disturb the uptake and translocation of other ions.

From this we may attempt to define at least three properties of a hypothetical calcifuge plant growing in an acid soil:

(1) An ability to thrive in situations where available phosphorus is at a low level, and in the face of phosphorus fixation by aluminium at the root surface.

(2) An ability to grow in low concentrations of calcium and in the face of impaired calcium uptake and translocation.

(3) The possession of either specific sites within the cytoplasm where aluminium may be harmlessly accumulated, or a specific method for chelating aluminium, and/or

(4) An ability to prevent aluminium from entering the cell by precipitation at the cell surface.

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